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On the Origin of Terpenes in Symbiotic Associations between Marine Invertebrates and Algae (Zooxanthellae)

CULTURE STUDIES AND AN APPLICATION OF $^{13}C/^{12}C$ ISOTOPE RATIO MASS SPECTROMETRY*

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$^{13}C/^{12}C$ ratios of sets of compounds, algal sterols and terpenes, isolated from dinoflagellate symbiont (zooxanthellae)-bearing soft corals and gorgonians were determined. In most cases, a significant difference was found between the $\delta^{13}C$ values of the terpenes and of the algal sterols from the same set, the algal sterols containing less ^{13}C than the terpenes. These results can only be explained if terpenes are synthesized by the host. Cultured zooxanthellae, isolated from symbiotic associations, do not make terpenes. Algal sterols of the various sets do not all have the same $\delta^{13}C$ value: average values range from −18.2 to −24.3‰. A consistent difference of about 7‰ in the $\delta^{13}C$ values of sterols of cultured symbionts isolated from two of the gorgonians was found. This has potential applications for the taxonomy of zooxanthellae, most of which are believed by some specialists to be one discrete species.

Many marine invertebrates, especially tropical coelenterates (*e.g.* stony and soft corals, gorgonians, sea anemones) living in shallow water, contain algal symbionts (1–4) which contribute to the nutrition of the host (5).

Chemists discovered that many of these marine invertebrates were rich sources of interesting natural products, *e.g.* terpenes (6–8) and prostanoids (9–11). Algae-bearing animals were also found to be the only source of two unusual sterols with cyclopropyl groups in the side chain, *viz.* gorgosterol (12) (Fig. 1) (*1i*) and 23-demethylgorgosterol (13) (*1g*). This raised the question of the origin of these sterols and natural products: were they made by the symbionts or by their host?

Corey and co-workers (14, 15) were unable to determine the origin of prostaglandins in the gorgonian *Plexaura homomalla*. More than a decade ago, Ciereszko *et al.* (16) reached the conclusion that gorgosterol (*1i*) was synthesized by the symbionts. Their experimental approach was questioned by Kokke *et al.* (17) and Withers *et al.* (18), but they confirmed the conclusion of Ciereszko *et al.* (16) that both cyclopropyl sterols are made by the algae (18). The origin of terpenes remained an open question. Dietary accumulation (8) of terpenes, as happens with some toxins (19), has been suggested. If this were the case, then many different filter and suspension feeders in the same location would contain the same terpenes, and this has never been observed. Also, terpenes are often present in large amounts in invertebrates, whereas no phytoplankter is known to produce terpenes.

The same method was used to attempt to solve the problem of the origin of the terpenes as was employed in the gorgosterol (*1i*) biosynthesis (17, 18). Terpenes were not detected in any of these cultured symbionts (zooxanthellae) which were studied for natural product content and sterol pattern. The reason might have been that the metabolic activity of symbiotic algae drastically changes when they are isolated and brought into culture. For example, cultured algae no longer excrete photosynthate (20, 21), and their sterol pattern is also affected: when living as symbionts they produce mainly Δ^5-sterols (*1*) required by their host (17); when living alone they produce 4-methyl sterols (*2*) as their main or only sterols (18).

There is direct evidence in the literature that some invertebrates are capable of terpene synthesis. Pukalide (*4*) and the structurally related cembrene lophotoxin (22) (*5*), other cembrenolides (23), and also furanodiene (24) (*3*) have been isolated from *Lophogorgia rigida*, *L. alba*, and *Pacifigorgia pulchra exilis* (gorgonians) from the Gulf of Mexico, and coraxeniolide A and related compounds (25) from *Corallium* sp. ("pink coral") from Hawaii, all of which lack symbionts. Pukalide (*4*) and furanodiene (*3*) have also been isolated from the soft corals *Sinularia abrupta* (26) and *Efflatounaria* sp. (27), respectively, which have symbionts. This did not eliminate the possibility that zooxanthellae, when living as symbionts, also synthesize terpenes. Thus, the problem was further investigated using $^{13}C/^{12}C$ ratios of compounds isolated from plants and animals.

$^{13}C/^{12}C$ variations are usually given as $\delta^{13}C$ values (for definition: see next section); the more negative this value, the less relative ^{13}C the sample contains. Because of isotope fractionation effects in photosynthesis (28, 29) it is easily possible to differentiate by the $\delta^{13}C$ values between terrestrial plants having the C_3 pathway (Calvin-Benson cycle) and the C_4 pathway (Hatch-Slack pathway) (30–33): the mode of $\delta^{13}C$ of C_4 plants is −13‰ and of C_3 plants −28‰.[1] Animals have slightly higher $\delta^{13}C$ values than their food (36, 37).

Because a reported $\delta^{13}C$ value (38) (−23.3‰) of zooxanthellae isolated from the mantle of the giant clam *Tridacna*

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[1] Seagrasses are a notable exception (34, 35).

maxima was significantly different from the $\delta^{13}C$ value (38) of the muscle of a related *Tridacna* sp.[2] (−16.0‰), we decided to apply isotope ratio mass spectrometry to the problem of the origin of terpenes in symbiotic associations. $\delta^{13}C$ values are not the same for all organic compounds from the same biological source. They depend on the biosynthetic pathway involved (*e.g.* they are different for lipids and sugars in plants) (40–45), but sterols and terpenes are related biosynthetically. Thus, if terpenes were made by the algae, we expected them to have the same $\delta^{13}C$ value as some sterols, known to be algal sterols,[3] isolated from the same symbiotic association: gorgosterol (*1i*), 23-demethylgorgosterol (*1g*), and 4-methyl sterols (*2*). The compounds whose $\delta^{13}C$ values we will discuss are not storage products, but metabolic end products; thus, isotope effects in catabolism affecting the $\delta^{13}C$ value of remaining uncatabolized material do not have to be taken into consideration.[4]

EXPERIMENTAL PROCEDURES

Isolation—References to papers describing the isolation of the terpenes are given in Table I; their structures are shown in Fig. 1. Procedures for making extracts and for the isolation of sterol mixtures are given elsewhere (47, 60). Sterols were separated by reverse-phase high performance liquid chromatography (eluent absolute MeOH) using Waters equipment and also a Valco CV-6-UHPa-N60 injector. Separation of most larger sterol samples involved use of an automated system (61) and a Whatman M9 10/50 ODS-3 column. Gorgosterol (*1i*) was obtained pure in this manner. Isolation of 24-methylenecholesterol (*1c*) by argentic silica gel TLC (62) from the sterol mixtures of *Sinularia* sp. and *Capnella imbricata* preceded ODS-3 fractionation. In the case of *Erythropodium caribaeorum*, this compound (*1c*) was isolated from an ODS-3 fraction by argentic silica gel TLC. Other ODS-3 fractions of interest were reinjected in a Whatman M9 10/50 ODS-2 column and pure cholesterol and mixtures enriched in 23-demethylgorgosterol (*1g*) were obtained. The latter compound was further purified by fractional crystallization from MeOH. The isolation of 4-methyl sterols (*2*) from three of the gorgonians has been reported earlier (17, 47). For the separation of the 4-methyl sterols from the cultured zooxanthellae, we also used two Altex Ultrasphere ODS columns (5 μ, 10 mm i.d. × 25 cm) in series. All sterol samples were recrystallized from MeOH to remove high performance liquid chromatography column bleed. The purity of all sterol samples was checked by 360 MHz ^1H NMR and/or gas chromatography (Hewlett-Packard model 402 gas chromatograph with a flame ionization detector, 3% SP 2250 column (2 mm i.d. × 1.80 m), 260 °C). The purity of terpene samples was determined using 360 MHz ^1H NMR.

Culture of Algae and Terpene Analysis—The isolation of the algae and the conditions under which they were mass-cultured were reported in another paper (17). Sterol, terpene, and acetogenin analysis were performed for the whole cells and the culture media for three gorgonian isolates (*Briareum asbestinum*, *Gorgonia mariae*, and *Muriceopsis flavida*). The extracts were carefully fractionated on silica gel and each fraction was analyzed by 220 MHz ^1H NMR. Known terpenoids, isolated from the whole animals, were utilized as standards and comparisons were made based upon R_f values by TLC and high performance liquid chromatography. Resonances at uncongested chemical shifts were utilized to question the presence of terpenes and acetogenins in the zooxanthellae (media) extracts. In no instance were any of the gorgonian-derived standards found in the zooxanthellae extracts. The cultured zooxanthellae isolated from *Oculina diffusa* (hard coral), *Tridacna gigas* (giant clam), *Melibe pilosa* (nudibranch), *Zoanthus sociatus* (zoanthid), and *Aiptasia pulchella* (sea anemone) were also analyzed for sterols (18) and terpenes, but terpenes were not detected. In these cases, terpenes isolated from the whole animals were not available as standards.

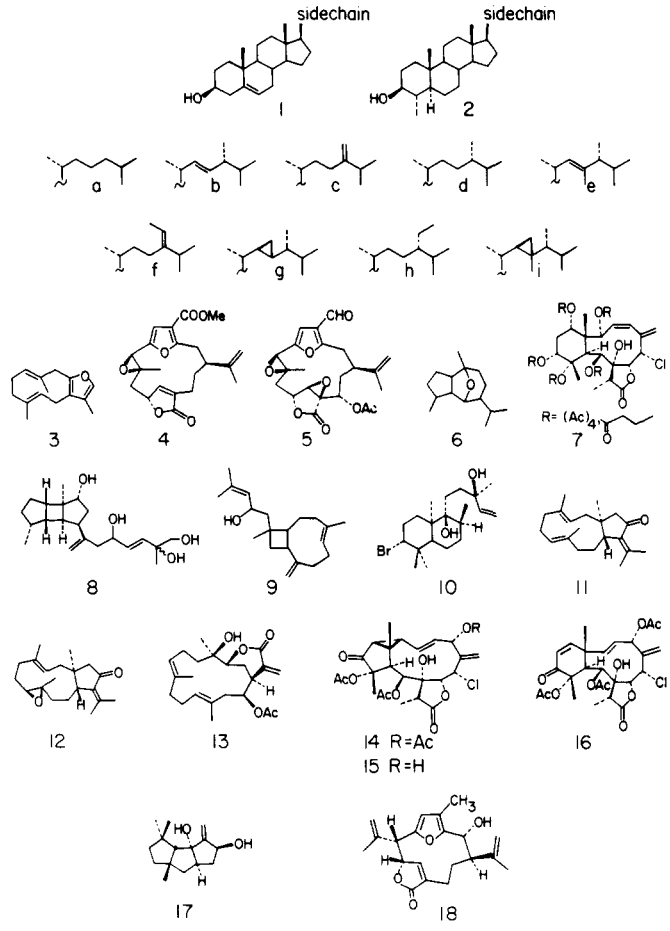

FIG. 1. **Structures of all compounds listed in Table I.**

$^{13}C/^{12}C$ Analysis—Oxygen gas was cycled over the sample at 800 °C in a vacuum combustion line and the CO_2 was removed using a liquid N_2-cooled trap. The CO_2 was used in a spectrometer for $\delta^{13}C$ analysis.

$$\delta^{13}C\ (‰) = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1\right] \cdot 1000$$

The standard is the carbonate of the Peedee belemnite. Routinely, the precision of this type of analysis is ±0.1‰.

RESULTS AND DISCUSSION

In Table I are listed all compounds which were analyzed, their sources, and the results of the analysis. They are terpenes, sterols known to be produced by the symbiotic algae (23-demethylgorgosterol (*1g*), gorgosterol (*1i*), 4-methyl sterols (*2*)), 24-methylenecholesterol (*1c*), and cholesterol (*1a*). Because cholesterol (*1a*) is the most abundant sterol in the marine environment, cholesterol (*1a*) is the main sterol (typically >80%) of filter and suspension feeders which do not have algal symbionts and which just accumulate dietary sterols (17). The sterol pattern is much different when the invertebrate has symbiotic algae which produce sterols: then cholesterol (*1a*) usually is only a minor sterol. 24-Methylenecho-

[2] $\delta^{13}C$ values of zooxanthellae isolated from three Caribbean stony corals are much higher: they range from −11.9 to −18.0‰ (39).

[3] Our experimental results show that this working hypothesis is only strictly true in the case of unicellular organisms. For details, see last section of discussion. Assume that farnesyl pyrophosphate (C_{15}) is a crossroads intermediate which is converted into a sesquiterpene (C_{15}) or dimerizes to form squalene and, eventually, a sterol. Even if the sterol and the sesquiterpene are synthesized in the same organelle of the same cell at the same time, there still would be a difference between the $\delta^{13}C$ value of the sterol and of the terpene. This difference would be small because the pathways diverge late in the synthesis and isotope fractionation might be large only at one or two of the 15 carbon atoms undergoing reaction.

[4] There is a rapid turnover of monoterpenes in some higher plants. Because of isotope fractionation in catabolism, one might expect a newly synthesized monoterpene molecule to have a $^{13}C/^{12}C$ ratio different from that of the existing pool of that terpene in the plant (46).

TABLE I

Sterols and terpenes, their sources and $\delta^{13}C$ values

Known algal compounds are in parentheses.

Symbiotic associations and compounds isolated therefrom	$\delta^{13}C$	Algae or animals without symbionts and compounds isolated therefrom	$\delta^{13}C$
	‰		‰
Efflatounaria sp.[a]		*Pacifigorgia pulchra exilis*[b]	
Furanodiene (*3*) (27)	−12.8	Furanodiene (*3*) (24)	−20.5
Sinularia sp.[a,5]		*Lophogorgia rigida*[b]	
Pukalide (*4*) (26)	−14.0	Pukalide (*4*) (22, 26)	−22.8
Cholesterol (*1a*)	−19.9	Lophotoxin (*5*) (22)	−20.8
(24-Methylenecholesterol) (*1c*)	−17.3		
(Gorgosterol) (*1i*)	−18.9		
Gorgonia mariae[b] (17, 47)		*Gorgonia mariae zooxanthellae* (18)	
"Sesquiterpene ether" (*6*) (48)	−18.5	(Cholesterol) (*1a*)	−15.2
Cholesterol (*1a*)	−18.4	(4α,24*S*-Dimethylcholestanol) (*2d*)	−11.2
(23-Demethylgorgosterol) (*1g*)	−19.2	(4α,24*R*-Dimethyl-22*E*-dehydrocholestanol) (*2b*)	−15.4
(4α,24*R*-Dimethyl-22*E*-dehydrocholestanol) (*2b*)	−17.8	(Dinosterol) (*2e*)	−15.2
Briareum asbestinum[b] (17, 47)		*Briareum asbestinum zooxanthellae* (18)	
Briarein B (*7*) (49, 50)	−18.2	(Dinosterol) (*2e*)	−20.7
Cholesterol (*1a*)	−19.9	(4α,24*S*-Dimethylcholestanol) (*2d*)	−22.6
(23-Demethylgorgosterol) (*1g*)	−24.3	(4α-Methyl-24*S*-ethylcholestanol) (*2h*)	−22.7
(Gorgosterol) (*1i*)	−24.7		
(4α-Methylgorgostanol) (*2i*)	−24.0		
Xenia macrospiculata[a]		*Stoechospermum marginatum*[c]	
Xeniaphyllenol (*9*) (51)	−15.2	(5*R*,15,18(*S* + *R*),19-Tetrahydroxy-spata-13,16*E*-diene) (*8*) (57)	−21.4
(Gorgosterol) (*1i*)	−23.1	(Fucosterol) (*1f*)	−15.7
Eunicea calyculata[b]		*Laurencia snyderae*[c] (58)	
13-Keto-1*S*,11*R*-dolabell-3*E*,7*E*,12(18)-triene (*11*) (52)	−17.4	(Concinndiol) (*10*) (59)	−16.2
7*S*,8*S*-Epoxy-13-keto-1*S*,11*R*-dolabell-3*E*,12(18)-diene (*12*) (52)	−17.9	(Cholesterol) (*1a*)	−18.9
Cholesterol (*1a*)	−21.0		
(23-Demethylgorgosterol) (*1g*)	−23.3		
(Gorgosterol) (*1i*)	−22.8		
Pseudoplexaura wagenaari[b] (47)			
Crassin acetate (*13*) (53)	−16.4		
Cholesterol (*1a*)	−20.4		
(23-Demethylgorgosterol) (*1g*)	−24.4		
(Gorgosterol) (*1i*)	−22.9		
(Dinosterol) (*2e*)	−22.6		
Erythropodium caribaeorum[b]			
Erythrolide A (*14*)[6]	−16.2		
Deacetylerythrolide A (*15*)[6]	−16.0		
Erythrolide B (*16*)[6]	−15.7		
Cholesterol (*1a*)	−20.5		
(24-Methylenecholesterol) (*1c*)	−21.0		
(Gorgosterol) (*1i*)	−22.6		
Capnella imbricata[a] (54)			
$\Delta^{9(12)}$-Capnellene-8β,10α-diol (*17*) (55)	−14.8		
(24-Methylenecholesterol) (*1c*)	−26.1		
(Gorgosterol) (*1i*)	−22.5		
Pseudopterogorgia kallos[b]			
"Pseudopterane (56) derivative" (*18*)[7]	−18.6		
Cholesterol (*1a*)	−21.4		
(Gorgosterol) (*1i*)	−22.1		

[a] Soft coral.
[b] Gorgonian.
[c] Seaweed.

lesterol (*1c*) is by far the main sterol of many soft corals from the Indo-Pacific (*e.g. C. imbricata* and *Sinularia* sp., 60.0 and 60.6% of the demethyl sterols, respectively) which suggested that it also was an algal sterol. This is now also suggested by its $\delta^{13}C$ value which is about the same as that of gorgosterol (*1i*) from the same source. 24-Methylenecholesterol (*1c* is also a major sterol (12.7%) of the gorgonian *E. caribaeorum*. Again, its $\delta^{13}C$ value indicates that it is produced by the zooxanthellae.

Samples of cholesterol (*1a*) from different organisms were analyzed to determine their $\delta^{13}C$ values. There are two possible origins of cholesterol (*1a*) in a symbiotic association. It must be made entirely by the zooxanthellae if the algae provide all organic nutrients needed to sustain their host. If the hosts also feed, at least part of the cholesterol (*1a*) should be of dietary origin. In this connection, it is important to point out that the invertebrates listed in Table I are known or supposed to be incapable of *de novo* sterol synthesis (63); thus, a $\delta^{13}C$ value of cholesterol (*1a*) identical with those of

[5] A. Sato, W. Fenical, and J. Clardy, Scripps Institution of Oceanography, and Cornell University, unpublished results.

[6] Look, S. A., Fenical, W., van Engen, D., and Clardy, J. (1984) *J. Am. Chem. Soc.*, in press.

[7] S. A. Look, W. Fenical, S. Rafii, and J. Clardy, unpublished results.

the algal sterols might be an indication that all cholesterol (*1a*) is made by the zooxanthellae and, hence, that the association is autotrophic. The data for *Gorgonia mariae* suggest that this symbiotic association is autotrophic whereas the other associations, from which cholesterol was isolated, are not autotrophic.

The $\delta^{13}C$ values of the terpenes of eight of the nine sets in Column 1 (Table I) are higher than the values of the algal sterols in the same set. The largest difference (7.9‰) is between gorgosterol (*1i*) and xeniaphyllenol (*9*) from the soft coral *Xenia macrospiculata*, and the lowest difference (3.5‰) is between gorgosterol (*1i*) and the pseudopterane derivative (*18*) from the gorgonian *Pseudopterogorgia kallos*. Those differences are real because we made every effort to make the samples completely pure. We expected terpenes and algal sterols to have the same $\delta^{13}C$ value only if the terpenes were also made by the zooxanthellae. Thus, we interpret our results as excellent evidence that in these eight symbiotic associations terpenes are synthesized by the host.

We tried to find an explanation for the fact that the terpenes in these sets have a higher $\delta^{13}C$ value than the algal sterols. Two sources of carbon are available to the host: dietary carbon and photosynthetic carbon (20, 21) produced by the zooxanthellae. $\delta^{13}C$ values of plankton (the source of food) indicate that the diet of *L. rigida* and *P. pulchra exilis* (collected in Pacific Mexico at about 24 °N) has a $\delta^{13}C \sim -21‰$ (64). Apparently, the $^{13}C/^{12}C$ ratio changes little when an animal uses dietary carbon to synthesize terpenes. More specifically, pukalide (*4*) and structurally related lophotoxin (*5*) and furanodiene (*3*) from these gorgonians, which do not have zooxanthellae, have similar $\delta^{13}C$ values as the diet of the gorgonians. These values are much lower, however, than the $\delta^{13}C$ value of pukalide (*4*) and furanodiene (*3*) isolated from the soft corals *Sinularia* sp. and *Efflatounaria* sp., respectively, which have symbionts (see Table I). Because dietary carbon is not fractionated (see above), the difference between the $^{13}C/^{12}C$ ratio of the terpenes and the algal sterols from the same set must be caused by significant differences in isotope fractionation effects in the conversion of photosynthetic carbon (20, 21) into terpenes by the host, and in sterol synthesis by the zooxanthellae. Mevalonate is the building block of terpenes and sterols. It is produced from three molecules of acetyl-CoA (65), which, in turn, is formed via decarboxylation of pyruvate. This decarboxylation reaction has been investigated and a large isotope effect was found (66, 67): lipids (*e.g.* sterols and terpenes) are depleted in ^{13}C as compared with the pyruvate from which they are derived. Pyruvate is a crossroad intermediate in biosynthesis: it can also be used, as such, for the synthesis of other compounds. It is important to note (66) that if all pyruvate were decarboxylated the acetyl group of acetyl-CoA would have the same isotopic composition as pyruvate.[8] If only a low percentage of the pyruvate were used for lipid synthesis, then the $\delta^{13}C$ value of the lipids would be much lower than that of the pyruvate (66). Thus, a possible explanation for the difference between the $^{13}C/^{12}C$ ratio of terpenes and algal sterols from the same set is that the host converts a much higher percentage of its pyruvate into acetyl-CoA than the zooxanthellae do. Alternatively, the pyruvate synthesized by the photosynthetic algae might already have a lower $\delta^{13}C$ value than the pyruvate made by their host from dietary carbon and algal photosynthate (20, 21).

Blair *et al.*[9] found that, when *Escherichia coli* is grown aerobically with glucose as its sole carbon source, acetate strongly enriched in ^{13}C ($\delta^{13}C$ +12.7‰) relative to glucose ($\delta^{13}C$ 0.0‰) is excreted into the medium. Indications are that most of the fractionation occurs at the stage of acetyl-CoA which can be converted into fatty acids ($\delta^{13}C$ −3.1‰) or citrate ($\delta^{13}C$ 0.0‰) which enters the Krebs cycle, or via acetylphosphate into acetate, which is excreted. Because of these results, we must also consider the possibility that glycerol (the main organic compound excreted by symbiotically living dinoflagellates (20, 21)) might have a $\delta^{13}C$ value different from that of pyruvate in the same algae even though both compounds would be derived from glyceraldehyde 3-phosphate, assuming that the algae are C_3 plants (68). Thus, isotope fractionation downstream from glyceraldehyde 3-phosphate but upstream from pyruvate might be a contributing factor to the observed differences in $\delta^{13}C$ values of algal sterols and terpenes from the same set (Table I).

The $\delta^{13}C$ values of the sesquiterpene ether and of the algal sterols of the gorgonian *G. mariae* are similar, which might mean that the terpene is synthesized by the algae. But the alternate explanation is that the terpene is synthesized by the host, as in the cases of the other eight symbiotic associations, but we cannot tell this from a difference in the $\delta^{13}C$ values of the terpene and of the algal sterols because, in this special case, the acetyl part of acetyl-CoA has accidentally the same $\delta^{13}C$ value in both host and symbionts.

All algal sterols from a particular organism do not have exactly the same $\delta^{13}C$ value (see Table I). The largest differences are observed in sterols from the *G. mariae* zooxanthellae and in *C. imbricata*. 4α,24*S*-Dimethylcholestanol (*2d*) from the *G. mariae* zooxanthellae has a $\delta^{13}C$ value of −11.2‰ which is 4.0–4.2‰ higher than the $\delta^{13}C$ value of the other sterols from the same source. The difference between the $\delta^{13}C$ values of the two algal sterols from *C. imbricata* is also large: 3.6‰. These differences are likely to originate in isotope fractionation effects in the biosynthesis of the various side chains (*b–i*) with more carbon atoms than the cholesterol side chain (*a*). All carbon atoms in the cholesterol side chain (*a*) are derived from mevalonate; the additional carbon atoms in side chains (*b–i*) are derived from methionine (69). Reactions involved in the formation of side chains (*b–i*) are alkylation, dehydrogenation, and reduction. For example, in the *G. mariae* zooxanthellae, 4α,24*S*-dimethylcholestanol (*2d*) is the precursor of the two other 4α-methyl sterols (*2b, e*). The precursor is dehydrogenated (70) to give 4α,24*S*-dimethyl-22*E*-dehydrocholestanol (*2b*) which is then converted into dinosterol (*2e*) by alkylation at the double bond (70). A demonstrated significant isotope effect in dehydrogenation reactions (44) probably contributed to the differences in the $\delta^{13}C$ values of the remaining precursor (*2d*) and the reaction products (*2b, e*).[10] The carbon of the methyl group of methionine, which is transferred to a substrate in biomethylation reactions (68), apparently has a $^{13}C/^{12}C$ ratio not much different from that of mevalonate carbons from the same organism as 4α,24*S*-dimethylcholestanol (*2d*) (which has one methionine-derived carbon atom) and dinosterol (*2e*) (which has two methionine-

[8] This might hold only for pyruvate produced by the symbionts from sugars (Embden-Meyerhof pathway) (66) or by the host from algal glycerol and sugars (20, 21) because photosynthetic carbohydrates seem to be isotopically homogeneous (66). No information is available on the $\delta^{13}C$ values of the carbons of pyruvate produced by animals from dietary carbon.

[9] N. Blair, A. Leu, E. Muñoz, J. Olsen, and D. Des Marais, NASA-Ames Research Center, and Stanford University, unpublished results.

[10] Isotope fractionation in the dehydrogenation reaction of *2d* cannot be the only explanation for the observed difference. The results of Monson and Hayes (44) show that one may expect a carbon fractionation factor of about 20 in a dehydrogenation reaction; a carbon fractionation factor resulting in a difference of 4‰ between the $\delta^{13}C$ values of *2d* and *2b,e* would be much higher. (The ratio of remaining precursor (*2d*) to products (*2b,e*) is 11:41 (17).)

derived carbon atoms) from the *G. mariae* zooxanthellae have very similar $\delta^{13}C$ values (Table I).

Table I clearly shows that the $\delta^{13}C$ values of the algal sterols from the symbiotic associations are not all the same: quite a large variation exists between the average values of each set. They range from −18.2‰ for *Sinularia* sp. to −24.3‰ for *B. asbestinum* and *C. imbricata*. Attempting to find an explanation for these differences, we analyzed sterols of the cultured symbionts of *G. mariae* and *B. asbestinum* (Table I, Column 2) and we found a consistent difference: the average values are −14.3‰ and −22.0‰, respectively. $\delta^{13}C$ values of algae are dependent on culture conditions (71) (*e.g.* pH, temperature) but both zooxanthellae were cultured under exactly the same conditions (17). The above difference between the $\delta^{13}C$ values of the sterols of the zooxanthellae is so large that, in the case of terrestrial plants, it would have been taken as an indication that different photosynthetic pathways were operating (30–33): the first value is compatible with a C_4 plant and the last value with a C_3 plant. The question which photosynthetic pathway is operating in each of the cultured algae cannot be answered. Very little is known about photosynthetic pathways in marine algae (72), but the reported $\delta^{13}C$ values for seaweeds and cultured algae (29, 38, 73, 74) do not fall into two distinct groups as values for terrestrial plants do (29–31). One reason might be that more or different photosynthetic pathways exist in marine algae then in terrestrial plants (72, 74). Another reason is that marine algae have two possible carbon sources: HCO_3^- and CO_2. Bicarbonate is enriched in ^{13}C as compared to CO_2 (75). The $\delta^{13}C$ value of CO_2 in the sea and in unpolluted air is about 7‰ lower than that of HCO_3^- in the sea. Thus, the difference in $\delta^{13}C$ values of the sterols of the cultured zooxanthellae might be due to a difference in the $\delta^{13}C$ values of their carbon sources. A carbon limitation (33) might also explain the observed differences. If both algae would differ only in their ablity to accumulate inorganic carbon from the medium (76–79) (all other variables being equal), then the alga with the most inorganic carbon available could be more isotopically selective than the other alga.

Algal taxonomists believe that one distinct species of dinoflagellate, *Zooxanthella microadriatica* (80) (= *Gymnodinium microadriaticum* = *Symbiodinium microadriaticum*) is found in most symbiotic associations with invertebrates (1–4) (with a possible exception of protozoans). Trench (81, 82), Kinzie (83) and their co-workers have challenged this classification by distinguishing among many strains of zooxanthellae which differ, *inter alia*, in their isozyme patterns, excretion rates, and compatability with various hosts. Our $\delta^{13}C$ values for the sterols of two different zooxanthellae strongly suggest that the isotopic composition of the different compounds might be a useful tool for the classification of zooxanthellae. We note that the two zooxanthellae had already been put into different groups because of large differences in sterol pattern (18).

The zooxanthellae from *B. asbestinum* and *G. mariae* produce sterols with a lower $\delta^{13}C$ value when they are living as symbionts than when they are cultured (the average values are −24.3 and −22.0‰, and −18.5 and −14.2‰, respectively). This difference is best explained if one assumes that algae, when living as symbionts, recycle (part of) the respired CO_2. As carbon sources, the symbiotic algae have respired CO_2 ($\delta^{13}C \sim -20$‰) and CO_2 ($\delta^{13}C \sim -7$‰) and HCO_3^- ($\delta^{13}C \sim 0$‰) from seawater (29). The cultured algae have only CO_2 and HCO_3^- from seawater available. In corals, $\delta^{18}O$ values of carbonates give nonequilibrium values suggesting lack of free exchange with the ocean bicarbonates (84, 85). Recycling of carbon can result in very low $\delta^{13}C$ values: *e.g.* −45.9‰ has been reported for the pognophore *Siboglinum atlanticum* which has symbiotic bacteria (86).

The working hypothesis that terpenes and sterols should have the same $\delta^{13}C$ value if they were synthesized by the same organism was put to a test. Fucosterol (*1f*) and a spatane derivative (*8*), isolated from the brown seaweed *Stoechospermum marginatum* from Ceylon, have significantly different $\delta^{13}C$ values: −21.4 and −15.7‰, respectively. A reason for this difference might be that brown algae are the most advanced seaweeds. Often, as in the case of the giant kelp, *Macrocystis majescula*, they are perennials, and it is feasible that the plant does not synthesize the terpene and sterol concurrently. Sterols are continuously synthesized because they are constantly needed as long as the seaweed grows. Metabolic changes resulting in variation in the $^{13}C/^{12}C$ ratio of newly synthesized compounds may occur as the plant ages. For example, metabolic changes, depending on the phase of its growth curve, have been reported for the unicellular alga *Dunaliella tertiolecta* (87). The red seaweed *Laurencia snyderae*, an annual plant, was also investigated. Again, the $\delta^{13}C$ values of the terpene, concinndiol (*10*), and cholesterol (*1a*), are not the same. The difference, however, between the two values is much lower than in the case of *S. marginatum*.

In multicellular plants, like these two seaweeds, there are many different types of cells. Photosynthate, part of it as storage products, is translocated to other parts of the plant. Isotope fractionation will occur when these storage products are utilized. Sterols and terpenes from plants should have similar $\delta^{13}C$ values if they are synthesized by the same cells and at the same time.[3] Also, the terpenes should be metabolic end products.[4]

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